THE INNERVATION OF HYPERPLASTIC EPIDERMIS IN THE MOUSE: A LIGHT MICROSCOPIC STUDY

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The innervation of the skin of hairless mice has been studied following induction of epidermal hyperplasia by physical and chemical methods. Physical stimuli comprised ultraviolet irradiation, heat, wounding, and friction. Effective chemicals included benzene, carbon tetrachloride, chloroform, creosote, formaldehyde, hexadecane, hydrobromic acid, sodium lauryl sulfate, and turpentine.

Epidermal hyperplasia, however produced, was associated with growth of sensory nerve fibers into the outer part of the epidermis. Following a single 10-min exposure to an ultraviolet sunlamp at 40 cm, the nerves extended into the epidermis within 24 hr and disappeared during the second week as the epidermis reverted to its normal thickness. Repeated irradiation (until tumors appeared) was accompanied by persistent hyperplasia and neural invasion. Of 32 papillomas examined, intraepithelial nerves were found in 28. The presence and location of nerves in the tumor epithelium were related to the incorporation of tactile hair disc epithelium.

The hyperplastic regenerative epithelium at the margins of skin ulcers were also invaded by nerves which sometimes followed the migrating epithelium across the ulcer floor. Since the regenerative epithelium was not directly treated, it was concluded that the proliferation of nervous tissue in response to skin injury was the result of the hyperplasia per se, regardless of the method used to produce it.

In mammalian skin, sensory and autonomic nerve fibers mingle in the cutaneous nerve plexus, which occupies the deep part of the dermis. From the plexus, sensory fibers ascend to terminate on hair follicles, in the connective tissue of the superficial dermis, and in the epidermis.

Two kinds of nerve endings are found in relation to the epidermis. In the first, an axon terminal penetrates the epidermal basement membrane and makes immediate contact with a Merkel cell. The structure and sensory function of this ending have been studied in detail, and these studies have been reviewed by Winkelmann and Breathnach [1]. The second, and relatively rare, ending is found between keratinocytes in the basal or spinous layer [2–4].

Induction of epidermal hyperplasia may result in alteration of the pattern of sensory innervation. When the epidermis of BALB mice is rendered hyperplastic by repeated applications of methylcholanthrene, the spinous and granular layers are invaded by sensory nerves [5]. Keratin stripping of human skin by means of Scotch tape results in penetration of the epidermis by nerve fibers within 24 hr; these fibers are very fine, and they appear to be lost within the following 24 hr [6]. In the rat, the epidermis which grows across the bed of a full-thickness skin wound also becomes invaded by nerve fibers [7].

In this paper we report a more detailed examination of the behavior of cutaneous nerves, as seen with the light microscope, following a variety of physical and chemical injuries to the skin.

MATERIALS AND METHODS

Hairless mice (Medical Research Council Research Laboratories, Carshalton, Surrey, England) were used throughout.

Physical Methods

Short-term irradiation with ultraviolet light (UVL). Anesthetized mice were placed in the supine position at a distance of 40 cm from a Westinghouse FS40 sunlamp with reflector. The lamp has a maximal emission of UV energy at 2800–3500 A. Each mouse was covered with a black polyethylene sheet containing four 3 × 3 mm cutouts through which the four quadrants of the abdomen were exposed. The holes were covered individually at the end of the selected exposure time, yielding four different exposure times per animal. Twelve of these skin areas were exposed for 8 sec (minimum) to 60 sec (maximum), 13 for 1.5 to 16 min, and 15 for 20 to 64 min. All 10 animals were killed on day 4 after irradiation.

Seven additional mice were irradiated in the same manner through two 3 × 3 mm cutouts for 10 min and the

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animals were killed on day 1 (24 hr), 2, 3, 4, 6, 10, or 18.

Long-term irradiation. Fourteen mice received whole-body UVL twice daily 5 days per week for 16 weeks, from the same source. During the first 4 weeks, dosage was increased from 10 min per session to 30 min per session. Individual mice were sacrificed when tumors appeared during the 20th to 25th weeks.

Heat. A 3-mm-diameter steel rod was heated to 70°C and its tip applied to the abdominal skin of 4 anesthetized mice for 1 to 8 sec. The animals were killed on day 4.

Keratin stripping. Scotch tape was applied to a 1-cm² area of the abdominal wall of 4 mice. It was gently peeled away after a few seconds, and applied repeatedly until horny material could no longer be seen readily on the tape (12–20 applications). The mice were killed on day 4.

Wounding. A full-thickness excised wound, 3 × 3 mm, was made on the abdominal skin of 4 anesthetized mice, which were killed on day 4.

Friction. A 1-cm² area of abdominal skin was abraded by means of fine-grade glass paper. It was found empirically that 10 strokes were sufficient to induce epidermal hyperplasia while avoiding ulceration. Two such animals were killed on day 4.

Chemical Methods

Two methods were used to restrict the area to which chemicals were applied, and to prevent rapid evaporation of volatile reagents: (1) Two microliters of reagent were pipetted onto a piece of glass coverslip measuring 3 × 3 mm and the glass was immediately inverted onto the abdominal skin. The glass was removed after 30 sec and the area dried with a cotton swab. The area was marked off with India ink and was treated in the same way on 3 successive days; the animals were killed on day 2 following the third application. The four quadrants of the abdominal wall were used in each animal to test the effects of successive dilutions of the reagents, which are listed in the Table. Saline (0.9%) was used as a control solution. (2) A 3-cm glass tube of 3-mm internal diameter was held upright on the abdominal skin of anesthetized mice. A hemostat was attached to a collar of filter paper around the tube, and rested on the table. The net pressure of the empty tube was 9 gm. Test reagent (0.5 ml) was injected into the tube. The area of contact with the skin was observed through an operating microscope, and any fluid diffusing at the edges was absorbed with filter paper. Reagents were applied to three quandrants of the abdominal wall in succession, for 1, 5, and 25 min, respectively. Saline (0.9%) was applied as a control to the fourth quadrant for 25 min. The solutions were then aspirated and the skin dried with a cotton swab. Acetone, absolute ethanol, chloroform, and ether were tested. The mice were killed on day 3.

Processing

From each animal a strip of skin was taken which included both the test area and adjacent untreated skin. The strips were mounted flat on filter paper and put in the following fixative: picric acid saturated in 90% ethanol, 70 ml; formaldehyde, 25 ml; glacial acetic acid, 5 ml. From the tumor-bearing mice, which had been subjected to long-term UVL treatment, 32 papillomas (27 from the dorsum, 3 from the abdomen, 2 from the face), together with the adjacent irradiated skin, were placed in the same fixative.

From each tissue block, 50 or more serial paraffin sections were taken at 15 μ and stained with a protargol method for nerve fibers [8]. The thickness of the epidermis in short-term UVL material was estimated by taking 10 random measurements of test and control areas (excluding stratum corneum) in each specimen.

TABLE. *Results of applying various chemicals to the skin of hairless mice on three successive days*

Reagent	Epidermal necrosis	Epidermal hyperplasia with neural invasion	No effect
Absolute ethanol			+
Acetone			+
Benzene		+	
Benzene 50% in acetone			+
Carbon tetrachloride 25% aqueous	+		
Carbon tetrachloride 10% aqueous		+	
Chloroform		+	
Creosote 100%	+		
Creosote 50% in acetone		+	
Formaldehyde 40%	+		
Formaldehyde 20% aqueous		+	
Hexadecane		+	
Hydrobromic acid	+		
Hydrobromic acid 50% aqueous	+		
Hydrobromic acid 25% aqueous		+	
Hydrobromic acid 10% aqueous			+
Sodium lauryl sulfate		+	
Turpentine		+	
Saline 0.9%			+

RESULTS

Normal Skin

The neurohistology of the skin of hairless mice has been described [9]. From the cutaneous plexus, nerve fibers ascend to terminate (a) on the epithelial remnants of tylotrich follicles; (b) in relation to groups of Merkel cells in the basal layer of the epidermis, i.e., in the tactile hair discs, or Haarscheiben [1], which lie immediately caudal to the tylotrich follicles; and (c) in a fine interfollicular subepidermal network. The fibers ascending to the tactile discs are rather coarse (2–4 μ) and are myelinated; they lose their myelin sheaths immediately prior to their terminal expansions beneath the Merkel cells. The interfollicular epidermal network is composed of very fine, unmyelinated axons. Nerve endings in relation to keratinocytes cannot be demonstrated with the light microscope, but Tsuji [4] has shown with the electron microscope that the tips of some axons may invaginate basal keratinocytes from below.

Ultraviolet Irradiation

A 10-min exposure elicited epidermal thickening which commenced within 24 hr and subsided

during the second week (Figs. 1–3). In the tactile discs, occasional axons extended into the spinous layer within 24 hr. During the next several days many axons could be found in the upper part of the epidermis of the discs. They pursued a straight or tortuous course among the spinous cells, sometimes dividing as they ran. The majority reached the stratum granulosum and a few appeared to terminate in the deeper part of the stratum corneum. At 10 days the number of intraepidermal axons was greatly reduced in comparison with the earlier material, and at 18 days none could be found. The position and orientation of the Merkel cells appeared to be unaffected. However, the expanded nerve terminals were reduced in number as invasion of the epidermis progressed. It was clear that the majority, at least, of the intraepidermal fibers were formed by the new growth of endings previously related to Merkel cells. In some of the 4-day material no expanded terminals were found; all the coarse ascending fibers had bypassed the Merkel cells to gain the spinous layer.

The interfollicular epidermis elsewhere participated in the hyperplastic response. However, it was not possible to demonstrate intraepithelial nerve fibers following short-term irradiation, except at the tactile discs. The minimum exposure required to elicit epidermal hyperplasia (as measured with an eyepiece micrometer on day 4) varied from 45 to 60 sec. Neural invasion of the tactile discs appeared to be inseparable from hyperplasia, i.e., it was not possible to elicit one response without the other.

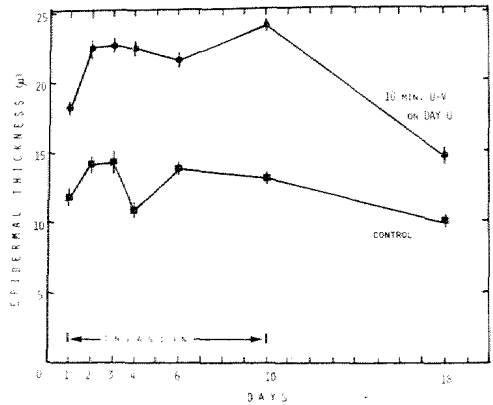

FIG. 3. Epidermal thickness (mean ± SE of 10 measurements) in irradiated and control skin areas of 7 mice. The observed duration of epidermal invasion by sensory nerves is indicated.

FIG. 1. Control tactile hair disc, showing two axons ascending through the dermis at center of field. The epidermis contains no nerve fibers (× 1000).

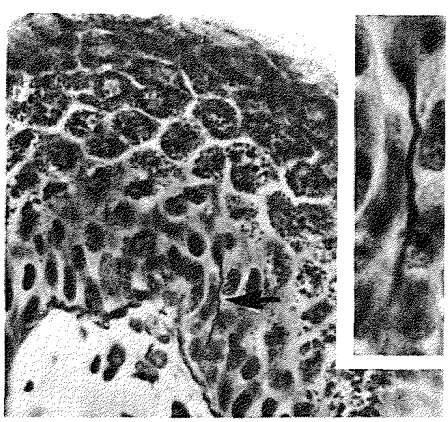

FIG. 2. Tactile hair disc on day 4 following 10 min of UVL. The epidermis is hyperplastic. An intraepidermal axon (*arrow*) is enlarged on the inset (× 1000, inset × 2500).

After 20 weeks of UVL treatment the epidermis of the trunk was 20 to 50 μ in thickness. The tactile hair discs were no longer structurally distinct because their thickness was similar to that of the epidermis elsewhere and because Merkel cells could no longer be identified. The location of the discs could nevertheless be ascertained by the presence of (a) persistent innervated remnants of tylotrich follicles (Fig. 4), and (b) groups of coarse intraepidermal axons in the epidermis immediately posterior to such follicles. Occasional isolated axons could also be found in the general interfollicular epidermis (Fig. 5).

The 32 tumors studied were all papillomas (Fig. 6). All were at early stages of development; none exceeded 3 mm in diameter and none showed evidence of invasiveness. Intraepithelial nerves were found in 28 of the tumors. Study of serial sections (including graphic reconstructions) showed that the nerves usually occurred in groups in one or two areas of the epithelium; the remainder of the epithelium was devoid of nerves. This clustering of intraepithelial fibers appeared at first to be random, but where tylotrich remnants were found underlying a tumor, it was the epithelium immediately posterior to the follicle which was invaded (Figs. 7, 8). The heavily innervated epithelial layers, therefore, seemed to be derived from tactile disc epithelium which had been incoporated into the tumor.

The cutaneous nerve plexus was incorporated to a variable extent in the pedicles of tumors. Some

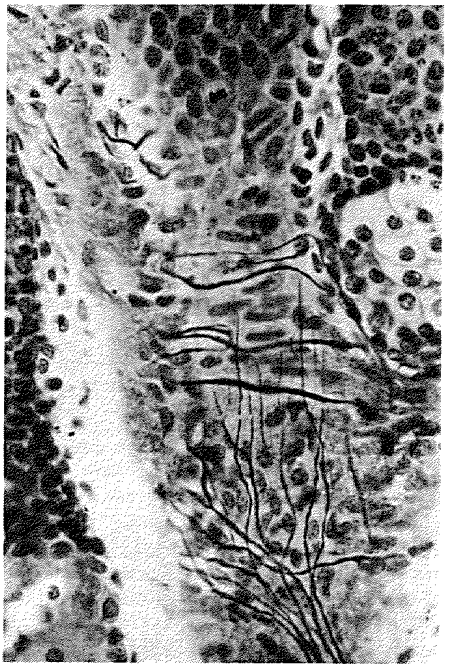

FIG. 4. Outer root sheath of tylotrich follicle showing normal pattern of innervation after long-term UVL. Remnants of smaller follicles are seen to left and right (× 600).

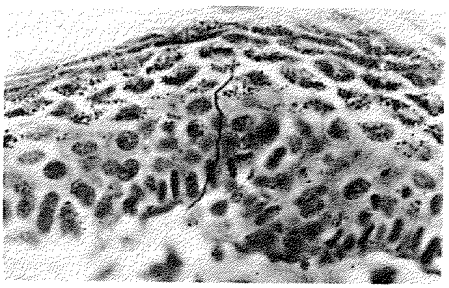

FIG. 5. Intraepidermal axon in long-term irradiated epidermis. There were no hair follicles in the immediate neighborhood (× 800).

histologic fields showed an abundance of nerve fibers here, but it was not clear whether this was the result of proliferation of the fibers or merely the inclusion of sections taken in the plane of the plexus whose orientation had been distorted by growth of the pedicle. Sections taken in the plane of the plexus would be expected to show a relatively large number of nerve fibers.

Other Physical Methods

The results of topical heat, friction, and keratin stripping were indistinguishable. Each resulted in pronounced hyperplasia, the epidermis being approximately doubled in thickness. The innervation of the epidermis on day 4 was comparable to that on day 4 following a 10-min exposure to ultraviolet irradiation.

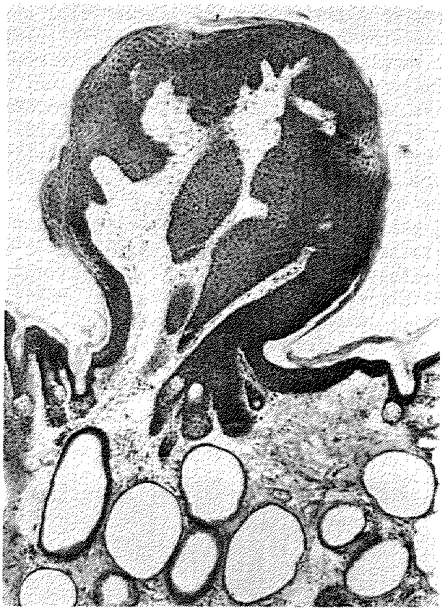

FIG. 6. Small papilloma from dorsal skin. The subcutaneous tissue contains sebaceous cysts (× 80).

FIG. 7. Portion of a papilloma showing tumor epithelium penetrated by a long nerve fiber (× 800).

FIG. 8. Remnant of a tylotrich follice underlying the epithelium shown in Figure 7. Note the persistent, heavy innervation (× 600).

The excised wounds were completely epithelialized on day 4. Both the repair epidermis and the untreated epidermis immediately adjacent were hyperplastic. Both of these hyperplastic zones contained occasional groups of nerve fibers. In the untreated zone the invading nerves were restricted to tactile disc epithelium, which was identified by adjacent tylotrich follicles.

The results of the coverslip method of application are summarized in the Table. There was a constant association between epidermal hyperplasia and neural invasion of the tactile discs. However, the quantitative relationships were quite unpredictable. For example, hydrobromic acid (50%) and formaldehyde (20%) produced a threefold increase in epidermal thickness but resulted in only a moderate epidermal invasion, approximately 4 axons being found in the epidermis of each tactile disc. Chloroform (100%) doubled the thickness of the epidermis; the tactile discs were filled with axons (Fig. 9). Sodium lauryl sulfate and hexadecane also doubled the epidermal thickness, but the neural response was only moderate.

By the glass-tube method, absolute ethanol, acetone, and saline had no detectable effect on the epidermis (on day 3), even after application for 25 min. On the other hand, chloroform and diethyl ether produced epidermal necrosis, together with extensive damage to the underlying dermis, after treatment for only 1 min. The nerve fibers in the superficial dermis were destroyed, and the repair epithelium migrating from the margins of the ulcer was not invaded from below. The untreated epidermis surrounding the ulcers underwent hyperplasia, and epithelial sheets migrated over the ulcer surface. Tactile discs close to the ulcer margins participated in the hyperplastic response, and they were deeply penetrated by nerve fibers. Occasionally the disc epithelium became part of a migrating epithelial sheet and the contained nerve fibers assumed a horizontal position (Fig. 10) and attained a length of up to 200 μ.

DISCUSSION

The methods used in this study, although not exhaustive, have been sufficient to show that epidermal hyperplasia, whether physically or chemically induced, is associated with epidermal invasion by sensory nerve fibers. In the short-term material the detectable invasion has been confined to tactile discs. Following long-term UVL treatment, nerve fibers could also be found in the general interfollicular epidermis. Although it has not been possible to determine by light microscopy whether fine fibers enter the general epidermis in the short-term material, it is reasonable to infer that they do enter, and that a period of maturation is required before they are sufficiently large, or impregnable with silver, to become visible.

The method of application of test reagents is important in determining the nature of the response. Application of chloroform under a coverslip for 30 sec on 3 successive days produced a

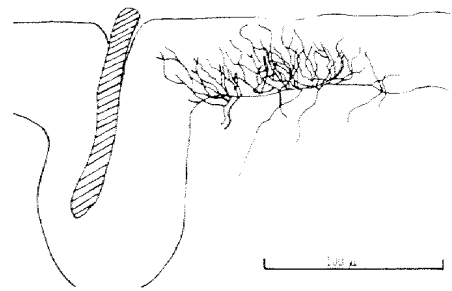

FIG. 9. Graphic reconstruction of five serial sections showing dense innervation of tactile disc epithelium following treatment with chloroform. Forty nerve fibers were counted in the epidermis. The hair (*shaded*) of the adjacent tylotrich follicle had not been shed; the nerve supply to this follicle is not included in the reconstruction.

FIG. 10. Horizontally disposed intraepidermal fibers following treatment with ether (× 1500).

moderate epidermal hyperplasia with densely innervated tactile discs. On the other hand, a single application of chloroform for 1 min in a glass tube led to complete epidermal necrosis. Using the coverslip method, benzene was as effective as chloroform in inducing hyperplasia. In a previous study using BALB mice [5] benzene was applied to the hairy skin by means of a cotton swab. Treatment twice weekly elicited only a 20 to 30% increase in epidermal thickness after several weeks, without any detectable change in cutaneous innervation. Species differences may provide a partial explanation for the relatively slight response of BALB mice, but the intimacy and duration of contact of the test reagent with the epidermis are probably of primary importance.

Both the precise mechanism and the functional significance of the growth of nerve fibers into the hyperplastic epidermis are obscure. The speed of the response is remarkable. At 24 hr after a single exposure to UVL, nerve terminals can be found in the outer part of the spinous layer. The earliest changes in the neuroepithelial relationships at the base of the epidermis are likely to be much earlier than this, and may well occur in advance of the initial burst of mitotic activity among the basal keratinocytes. Cajal [10] postulated that trophic factors in the epidermis could cause invasion by nerve fibers during normal ontogeny. It is of interest here that the epidermis of digital skin in the human [11] and monkey [12] fetus is extensively penetrated by nerve fibers which are subsequently lost. The same phenomenon has been seen in the rat and mouse, in which the epidermis is heavily stratified and is deeply penetrated by nerve fibers in the perinatal period [13]. Indeed, the histologic appearances of the hyperplastic, innervated epidermis in the present study are reminiscent of newborn mouse skin. Fetal epidermis and regenerating epidermis are both immature, and in order to account for their similar innervation it has been suggested that ingrowth of nerve fibers into epidermis may be a release phenomenon due to the absence from immature epidermis of a hypothetical deterrent to nerve growth (7).

In chemically induced hyperplasia, obvious differences were observed in the numbers of intraepidermal fibers between one successful chemical and another. We have been unable to account for these differences, which could not be explained by technical differences among the several experiments.

The discovery of intraepithelial nerves in papillomas does not appear to have special significance. The nerves are in general longer than those of the adjacent hyperplastic epidermis; their growth, both in the pedicle and in the tumor epithelium, keeps pace with the expansion of the connective tissue and epithelial elements of the tumors. The density of innervation of papillomas appears to be fortuitous, being determined primarily by the number of tactile discs which participate in a given tumor. The picture observed in the present study was different from that of Pawlowski and Weddell [14] in their study of human basal cell epitheliomas. These workers noticed a multiplication of neural elements which lay in contact with the tumor cell aggregates but which did not penetrate them. The appearances suggested the formation of a "barrier" of neural elements attempting to stem the tide of invading cells. The present observations are not in conflict because our material did not include tumors at an invasive stage.

The presence of nerves in the hyperplastic epithelium adjacent to a wound is of particular interest. They are found adjacent to excised wounds and other ulcers produced by ether or chloroform. Since the epidermis concerned has not been injured directly, it is reasonable to deduce that the invasion of the epidermis is the result of the hyperplastic response per se, regardless of the method by which the hyperplasia is produced.

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